Appendix B

Engineered Zinc Finger–Activating Vascular Endothelial Growth Factor Transcription Factor Plasmid DNA Induces Therapeutic Angiogenesis in Rabbits With Hindlimb Ischemia

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Background—Therapeutic angiogenesis seeks to promote blood vessel growth to improve tissue perfusion. Vascular endothelial growth factor (VEGF) exists in multiple isoforms. We investigated an engineered zinc finger–containing transcription factor plasmid designed to activate the endogenous VEGF gene (ZFP-VEGF).

Methods and Results—New Zealand White rabbits (n=56) underwent unilateral femoral artery ligation and excision. At day 10 postoperatively, the ischemic muscle received ZFP treatment (500 μg ZFP-VEGF plasmid) or no ZFP treatment (β-galactosidase, empty, or no plasmid). Group 1 (n=13) was harvested 3 days after injection to examine VEGF mRNA by real-time polymerase chain reaction and protein by ELISA. Groups 2 (n=13) and 3 (n=10) were harvested 11 days after injection. Group 2 was studied by histology and group 3, by histology and changes in blood flow. Groups 4 and 5 (n=10 each) were harvested 22 and 32 days after injection, respectively, and studied for changes in blood flow. In group 1, VEGF mRNA copy numbers were significantly higher for $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and protein in the ZFP-VEGF-treatment versus no-ZFP-treatment arms. In groups 2 and 3, capillary density and proliferating cells were significantly greater and apoptosis significantly lower in the treatment versus no-treatment arms. Changes in the blood flow ratio of the ischemic to the nonischemic limb were significantly greater in the treatment versus no-ZFP-treatment groups (6.57±1.52% versus 3.38±0.87%, $P<0.005$; 13.15±1.77% versus 6.13±1.55%, $P<0.001$; and 20.16±2.84% versus 13.88±3.14%, $P<0.01$, for groups 3, 4, and 5, respectively).

Conclusions—This engineered ZFP-VEGF–activating transcription factor may provide a novel approach to treat peripheral arterial disease. (***Circulation.*** 2004;110:2467-2475.)

Key Words: muscle ■ angiogenesis ■ growth substances ■ apoptosis ■ endothelium-derived factors

Peripheral arterial obstructive disease (PAOD) due to atherosclerotic vascular disease is a major health problem in the United States. The 2 primary clinical manifestations of PAOD are intermittent claudication and critical limb ischemia. With intermittent claudication, there is leg pain or aching with exercise that is relieved with rest, and in critical limb ischemia, there is rest pain, ischemic ulcers, or gangrene.[1] Collectively, PAOD has an age-adjusted prevalence of 12%, and the number of patients with PAOD will increase as the population ages.[1–3] Although the primary pathophysiology of PAOD is obstructive atherosclerosis leading to impaired perfusion in the lower extremities, treatment strategies in patients with PAOD are directed toward modifying the underlying pathophysiological etiologies.[4] Surgical or percutaneous revascularization strategies may be able to improve blood flow in selected patients with PAOD, but a large number of patients with PAOD are not eligible or are poor candidates for revascularization. Currently, there are no medical treatments for PAOD designed to increase blood flow to the ischemic limb.

Angiogenesis is defined as the growth and development of new capillaries from preexisting vasculature, and therapeutic angiogenesis seeks to exploit this phenomenon for the treatment of disorders of inadequate tissue perfusion.[6,7] The exogenous administration of an angiogenic growth factor offers promise for patients with PAOD. Vascular endothelial growth factor (VEGF) is a prototypical angiogenic growth factor that stimulates angiogenesis in vitro and in vivo.[8]

TABLE 1. Summary of Groups Used in Analyses (N=56 Rabbits)

Group 1 (n=13): Harvesting at Day 3 After Injection; Total Duration 13 Days	Group 2 (n=13): Harvesting at Day 11 After Injection; Total Duration 21 Days	Group 3 (n=10): Harvesting at Day 11 After Injection; Total Duration 21 days	Group 4 (n=10): Harvesting at Day 22 After Injection; Total Duration 32 Days	Group 5 (n=10): Harvesting at Day 32 After Injection; Total Duration 42 Days
VEGF expression	...	...	...	...
...	Histology	Histology	Histology	Histology
...	Blood flow measurement (only at day 21 of ischemia before euthanasia)	Blood flow measurement at days 10 and 21	Blood flow measurement at days 10 and 32	Blood flow measurement at days 10 and 42
n=6 ZFP-VEGF treated (plasmid ZFP-VEGF/poloxamer)	n=8 ZFP-VEGF treated (plasmid ZFP-VEGF/saline)	n=5 ZFP-VEGF treated (plasmid ZFP-VEGF/poloxamer)	n=3 ZFP-VEGF treated (plasmid ZFP-VEGF/poloxamer)	n=5 ZFP-VEGF treated (plasmid ZFP-VEGF/poloxamer)
n=7 Control ("empty" ZFP plasmid)	n=5 Control (plasmid pCMV-β–gal/saline)	n=5 Control (plasmid pCMV-β–gal/saline and sham)	n=7 Control (empty plasmid and poloxamer only)	n=5 Control (saline only)

All rabbits received injection materials at day 10 of ischemia (amount of injected material in a total volume of 2 mL: 500 μg for plasmids). CMV indicates cytomegalovirus; β-gal, β-galactosidase. All other abbreviations are as defined in text.

Alternative splicing of the human VEGF mRNA produces at least 4 protein isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, that vary with respect to their solubility and affinity for binding to extracellular matrix.[8,9] All of these VEGF isoforms exist in vivo, and the various isoforms appear to be differentially regulated in the setting of ischemia.[10]

The delivery of a single isoform of VEGF has been used in a number of uncontrolled human clinical trials that reported exciting clinical results, although significant edema developed in the VEGF-treated limb.[11–13] VEGF has been used in only 1 placebo-controlled clinical trial in patients with PAOD. The RAVE trial used unilateral intramuscular administration of adenovirus encoding $VEGF_{121}$, and in that study, $VEGF_{121}$ was not associated with improved exercise performance or quality of life but was associated with increased peripheral edema.[14] Taken together, human investigations that have used a single isoform of VEGF have been disappointing.

A number of genes, including the VEGF gene, contain DNA sequences that serve as sites for the binding of endogenous zinc-finger transcription factors, and some of these zinc-finger binding sites have the potential to serve as sites that regulate gene expression in vivo.[15–18] Zinc-finger protein (ZFP) transcription factors can also be engineered to bind to specific DNA sequences, and by fusing the DNA binding protein to a transcriptional activator or repressor, these engineered transcription factors can be used to increase or decrease, respectively, gene expression. Rebar et al[16] showed that an engineered ZFP linked to a transcriptional activation domain was able to increase endogenous VEGF gene expression in normal mouse skeletal muscle and promote angiogenesis in a mouse ear model in vivo. When compared with the delivery of a single VEGF isoform, the potential advantages of a gene therapy approach with a ZFP transcription factor is that activation of the endogenous VEGF gene should produce multiple VEGF splice variants. However, it was unknown whether a VEGF-activating transcription factor could induce gene expression and favorably modulate the angiogenic response in ischemic muscle. To address this question, we used a rabbit hindlimb ischemia

model to test the effects of intramuscular delivery of a plasmid DNA encoding a ZFP-VEGF–activating transcription factor.

Methods

Plasmid DNA Constructs and Formulations

The VEGF-activating ZFP (mVZ+426) was provided by Sangamo BioSciences (Point Richmond, Calif) and has been previously described.[16] In brief, the genetically engineered plasmid encodes the designed 3-finger ZFP DNA-binding domain, the nuclear translocation signal from simian virus 40 large T antigen, and the transactivation domain from the p65 subunit of the human nuclear factor-κB, subcloned into pVAX1 (Invitrogen) with expression under direction of the cytomegalovirus promoter. An identical plasmid lacking the ZFP insert and a plasmid DNA encoding the β-galactosidase gene were also provided and previously described.[16] Vials containing the ZFP-VEGF plasmid, the identical plasmid without the insert, and the β-galactosidase were identical in appearance and were coded to maintain blinding until data analysis was complete.

Hindlimb Ischemia Model and Treatment Groups

In total, 56 female New Zealand White rabbits with surgically induced unilateral hindlimb vascular insufficiency were studied. By previously described methods,[10,19] rabbits (mean weight, 3.1 kg) were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). A longitudinal incision was made along the left medial thigh to allow proper isolation, ligation, and excision of the femoral artery from its origin just above the inguinal ligament to its bifurcation at the origin of the saphenous and popliteal arteries. The inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries were also isolated and ligated. The incision was closed and all animals were closely monitored during the postoperative period. Two investigators (Q.D. and J.H.) performed all of the surgical procedures.

After 10 days of ischemia, the rabbits were again anesthetized with ketamine and xylazine. For some groups (Table 1), bilateral hindlimb perfusion measures were performed, and these methods are described in a subsequent paragraph. At this time, all rabbits were divided into "ZFP-treatment" or "no-ZFP-treatment" arms. A separate 2-cm longitudinal incision was made along the ischemic limb to allow exposure of the anterior leg, tibialis anterior (TA) muscle belly. In all cases, 4 intramuscular injections were made with a 27G1/2 syringe needle evenly across the muscle. Each injection was performed smoothly for at least 15 seconds, and the needle was left in place for at least 10 seconds to prevent backflow of the injected material. The skin incision was then closed with 2-0 interrupted silk sutures (Ethicon). Group assignments were made to accomplish

different study objectives and therefore, had different lengths of time from dosing until study termination. Based on their group assignment (Table 1), the study was completed 3 days later (group 1, n=13), 11 days later (group 2, n=13, and group 3, n=10), 22 days later (group 4, n=10), or 32 days later (group 5, n=10). Within the aforementioned groups, the ZFP-VEGF–activating transcription factor was administered as 500 μg of plasmid in a total volume of 2 mL of 1% poloxamer or saline (150 mmol/L). The no-ZFP-VEGF treatment included several complementary methods: a plasmid encoding a cytomegalovirus β-galactosidase in saline (500 μg in a total volume of 2 mL), an "empty" plasmid that contained the same backbone as the ZFP-VEGF but no insert (500 μg in a total volume of 2 mL), poloxamer with no plasmid DNA (500 μg in a total volume of 2 mL), saline only (total volume 2 mL), and sham (needle-only) injections. A single investigator (Q.D.) who, with the exception of the 2 rabbits within group 3 in which no material was injected, was blinded to the "ZFP-VEGF treatment" or "no-ZFP-VEGF treatment" arm assignment, performed all of the treatments. All protocols and procedures involving animals conformed with the Guidelines for Use of Laboratory Animals published by the US Department of Health and Human Services and approved by the Duke University Animal Care and Use Committee. All animals received care in accordance with *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Tissue Procurement, Histological Section Preparation, and mRNA and Protein Extraction

After bilateral hindlimb perfusion measures were performed after light anesthesia was induced with ketamine and xylazine, additional anesthetic was given, the ischemic and contralateral nonischemic TA muscles were surgically excised from tendon to tendon, and the rabbits were euthanized after muscle extraction with a single intravenous dose of phenobarbital (150 mg/kg) and phenytoin sodium (25 mg/kg). The tissue sample was divided into several parts. The midportion of the muscle was placed in 30% sucrose–phosphate-buffered saline solution (PBS), mounted in cross section in OCT compound, and snap-frozen in LN_2. Cryostat sections (5 μm) were prepared on microscope slides (Superfrost Plus, Fisher Scientific) for histological analysis. The remainder was snap-frozen in LN_2 for RNA and protein extraction. Muscle samples were weighed and extracted with TRIzol total RNA isolation reagent (GIBCO-BRL). In brief, tissue samples were homogenized in 200 μL TRIzol reagent per 50 to 20 mg tissue and incubated for 10 minutes at room temperature; afterward, 40 μL chloroform per 200 μL of TRIzol reagent was added. After centrifugation at 13,000g for 20 minutes at 4°C, the RNA-containing colorless upper aqueous phase of the mixture was collected, precipitated, and washed with isopropyl alcohol and 75% ethanol. RNA concentration was determined by spectrophotometry. For protein determination, muscle samples were weighed, homogenized, and centrifuged, and the protein content of the supernatant was determined by Bradford assay by previously described methods.[10,19,20]

Measurement of VEGF mRNA and Protein in Skeletal Muscle

After extraction, the RNA was treated with DNase and purified with use of an RNeasy Mini kit (Quiagen) according to the manufacturer's instructions. One microgram of total RNA was used for first-strand cDNA synthesis by reverse transcription (RT) with Multi-Scribe reverse transcriptase and random-hexamer primers for 12 minutes at 42°C as described by the manufacturer's instructions (GeneAmp Gold RNA PCR reagent kit, AB Applied Biosystems). In total, 50 ng of cDNA products were amplified by quantitative TaqMan real-time RT–polymerase chain reaction (PCR) with a TaqMan Universal PCR Master Mix kit as described in the manufacturer's instructions (AB Applied Biosystems). A single-tube PCR was optimized for quantification of rabbit specific primers and probes for $VEGF_{121}$, $VEGF_{165}$,

TABLE 2. Summary of Groups Used in Analyses (N=56 Rabbits)

Primer and Probe Name (5'-3')	Sequence	Product S
VEGF$_{121}$		
Forward primer	GAGATGAGCTTCCTACAGCA	77 bp
Reverse primer	TCGGCTTGTCACATTTTCTTG	
Probe	TGTCTTTCTTTGGTCTGCATTCACA	
VEGF$_{165}$		
Forward primer	AGAGCAAGGCAAGAAATCCC	90 bp
Reverse primer	TGCAGGAACATTTACACGTCT	
Probe	AAATGCTTTCTCCGCTCTGAGCAA	
VEGF$_{189}$		
Forward primer	AGGAAAGGGCAAGGGGCAA	116 bp
Reverse primer	TCTGCGGATCTTGTACAAACA	
Probe	CCCACAGGGAACGCTCCAGGA	
Rabbit 18S rRNA		...
Forward primer	TTCCGATAACGAACGAGACTCT	
Reverse primer	TGGCTGAACGCCACTTGTC	
Probe	TAACTAGTTACGCGACCCCCGAG	

Abbreviations are as defined in text.

and $VEGF_{189}$ as shown in Table 2. A sequence detector (ABI Prism 7700, PE Applied Biosystems) was used to continuously measure the amplified product in direct proportion to the increase in fluorescence emission during the PCR amplification. All real-time RT-PCR data were captured with sequence detector 1.7 software (PE Applied Biosystems). For each sample, an amplification plot was generated. From each amplification plot, a threshold cycle (Ct) value was calculated, representing the PCR cycle number at which fluorescence was detectable above an arbitrary threshold. The target gene's mRNA concentrations were calculated with the Ct value and were normalized against 18S rRNA. Negative controls lacking template RNA were always included in each experiment. Each sample was tested in triplicate. Total VEGF protein concentrations were determined by a solid-state ELISA system with a Quantikine VEGF ELISA kit (R&D Systems) as previously described.[10]

Analysis of Capillary Density, Proliferation, and Apoptosis in Skeletal Muscle

Capillary density in skeletal muscle was measured by endogenous endothelial alkaline phosphatase staining on frozen sections by a previously described method.[10,19] In brief, slides were prefixed in acetone and incubated with nitroblue tetrazolium–5-bromo-4-chloro-3-indoylphosphate-p-toluidine salt (GIBCO-BRL) at room temperature for 1 hour. The slides were postfixed in 10% formalin and then counterstained with eosin. Capillaries appear dark blue against a red background. Capillary density expressed by the number of capillaries per square millimeter was measured by counting 6 random, high-power (magnification, ×200) fields or a minimum of 200 fibers from each (ischemic and nonischemic) limb on an inverted light microscope. Photographs were taken with an Optometrics analog camera and Adobe Premier Version 5.1, and these images were analyzed with an NIH Image analysis system. A hemocytometer was used to standardize area measurements. To ensure that the analysis of capillary density was not subjected to error from muscle atrophy or interstitial edema, vascular density was also determined by dividing the number of capillaries by the number of muscle fibers to yield the capillary–muscle fiber ratio.

Immunohistochemistry was performed with modifications of previously described methods.[19-21] Frozen sections were allowed to come to room temperature and placed in ice-cold acetone for 10

minutes and then in PBS for three 5-minute washes. Blocking solution (10% normal horse serum in PBS) was applied for 20 minutes at room temperature. To confirm the results of the alkaline phosphatase stains, an endothelial cell antibody (CD31, R&D Systems) was used at a final concentration of 1 μg/mL at 4°C overnight. Endothelial cells with adjacent pericytes or vascular smooth muscle cells were identified with an antibody against α-smooth muscle actin (HHF35, DAKO Corp) at a final concentration of 16.4 μg/mL at room temperature for 1 hour. To detect the fraction of proliferating cells, a mouse monoclonal anti-human proliferating cell nuclear antigen (PCNA) antibody (DAKO Corp) was used at a final concentration of 1 μg/mL at 4°C overnight. Incubation with the primary antibody was followed by sequential incubation with a biotinylated anti-mouse IgG and ABC reagent, according to the manufacturer's instructions (Vectastain ABC kit, Vector Laboratories). Levamisole was added to block endogenous alkaline phosphatase activity, and immune complexes were localized with the use of the chromogenic alkaline phosphatase substrate Vector Red (Vector Laboratories). All sections were counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific). For the PCNA study, antibody to human tonsil tissue was used as a positive control, and PBS was used in place of a primary antibody as a negative control.

An in situ nick end-labeling (NEL) apoptosis kit (ApopTag, Intergen Co) was used to label apoptotic cells in skeletal muscle of histological sections as described.[19,20] This method labeled the fragmented DNA with terminal deoxynucleotidyl transferase (TdT) with digoxigenin-conjugated peroxidase staining and a methyl green counterstain, as described in the manufacturer's instructions. Positive control slides were pretreated with DNase I (Sigma) at 100 ng/mL for 10 minutes at room temperature, followed by end-labeling. Negative controls were performed without active TdT. In selected samples, TUNEL staining was followed by immunohistochemistry to identify the apoptotic cell type.

For the PCNA staining and apoptosis studies, the proliferation index and apoptotic index were expressed as a percentage of the number of positive nuclei divided by the total number of nuclei. The count was performed on 3 randomly selected fields (magnification ×200) and at least 200 nuclei per sample. A single reader blinded to sample type performed all of the analyses. The variability on repeated measures was <10%.

Hemodynamic Assessment

Bilateral hindlimb perfusion was measured with VASAMEDICS LASERFLO blood perfusion monitor laser Doppler equipment (VASAMEDICS, Inc) after induction of anesthesia with ketamine and xylazine. Based on the group assignments shown in Table 1, blood flow was measured at the day of study termination or at both preinjection (10 days after ligation) and at 11, 21, or 31 days after injection, which correspond to a total of 21, 32, and 42 days of ischemia. In total, 6 sites from the upper, middle, and lower part of each hindlimb were selected for measurement for each limb. For all analyses, a mean value was calculated from all 6 measurements per limb.

Statistical Analysis

Unless otherwise stated, results are expressed as mean±SD, the only exception being for VEGF expression data, for which data are expressed as mean±SE. Statistical significance was evaluated by Student's *t* test for paired or unpaired variables. *P*<0.05 or less was considered statistically significant.

Results

Treatment With the ZFP-VEGF Plasmid Increased VEGF mRNA Expression and Protein Production in Ischemic Skeletal Muscle

First, we sought to determine whether the ZFP-VEGF plasmid was able to increase the expression of 3 different VEGF splice variants and VEGF protein. In total, 13 rabbits were harvested on day 3 after injection (total of 13 days of ischemia) from group 1 (Table 1). This ZFP plasmid dose was selected from studies performed in nonischemic rabbit muscle (J.R., unpublished data), and the 3-day postinjection time was selected because that was when the plasmid was expected to be expressed but before changes in muscle blood flow would occur, as the latter could influence VEGF expression. The no-ZFP-treatment arm used the plasmid without the ZFP-VEGF insert.[16] Expression of $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ mRNAs was measured by quantitative real-time RT-PCR and expressed as mRNA concentration in the ischemic limb minus the value in the nonischemic limb for each animal. As shown in Figure 1A (top), the differences were 5.65±2.79, 3.99±2.07, and 5.67±2.81 in the ZFP-VEGF–treated arm compared with 0.12±0.24, −0.20±0.19, and −0.26±0.19 in the no-ZFP-treatment arm for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ mRNAs (*P*=0.05, *P*=0.05, and *P*=0.04), respectively. VEGF protein was measured by ELISA and was expressed as concentration in the ischemic limb minus the nonischemic limb for each animal. As shown in Figure 1B (bottom), the difference in VEGF protein was 40.65±8.83 versus 9.06±8.15 pg/mg soluble protein (*P*=0.02) for the ZFP-treatment versus no-ZFP-treatment arms. The mRNA data were also analyzed as the difference in VEGF mRNA in the ischemic limb of the ZFP-treatment arm compared with the no-ZFP-treatment arm, and the values were 15.05±8.58 versus 1.80±0.27, 12.57±7.04 versus 1.20±0.27, and 10.19±5.0 versus 1.08±0.26 for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ mRNA, respectively. These differences were not statistically different; however, levels of VEGF protein were significantly greater in ischemic muscle in the ZFP-treatment arm versus the no-ZFP-treatment arm (88.9±10.75 versus 52.31±7.07 pg/mg soluble protein, *P*=0.01). Taken together, these data suggest that the ZFP-VEGF transcription factor was able to increase VEGF mRNA and protein expression in ischemic skeletal muscle.

ZFP-VEGF Plasmid Favorably Modulates Capillary Density, Cell Proliferation, and Apoptosis in Ischemic Skeletal Muscle

Next, we sought to determine whether the ZFP-VEGF plasmid was able to increase vascular density in ischemic skeletal muscle compared with no ZFP treatment 11 days after injection. Alkaline phosphatase staining was used to measure capillary density, and representative examples are shown in Figure 2A and 2B. As shown in Table 1, a total of 23 rabbits from groups 2 and 3 were available for quantitative analysis (n=13 in the ZFP-VEGF treatment arm and n=10 in the no-ZFP-treatment arm). As shown in Figure 2C, capillary density in the ischemic TA muscle was significantly higher in the ZFP-VEGF-treatment arms compared with the no-ZFP-treatment arm (310±71 versus 198±25 capillaries/mm², *P*<0.001). To exclude any potential differences induced by changes in muscle fiber diameter, the capillary–muscle fiber ratio in the ischemic TA muscle was measured. The capillary–muscle fiber ratio was also significantly higher in the ZFP-VEGF-treatment arm compared with the no-ZFP-treatment arm (1.08±0.15 versus 0.85±0.11, *P*<0.05). By CD31 immunostaining, capillary density was 1.9-fold higher
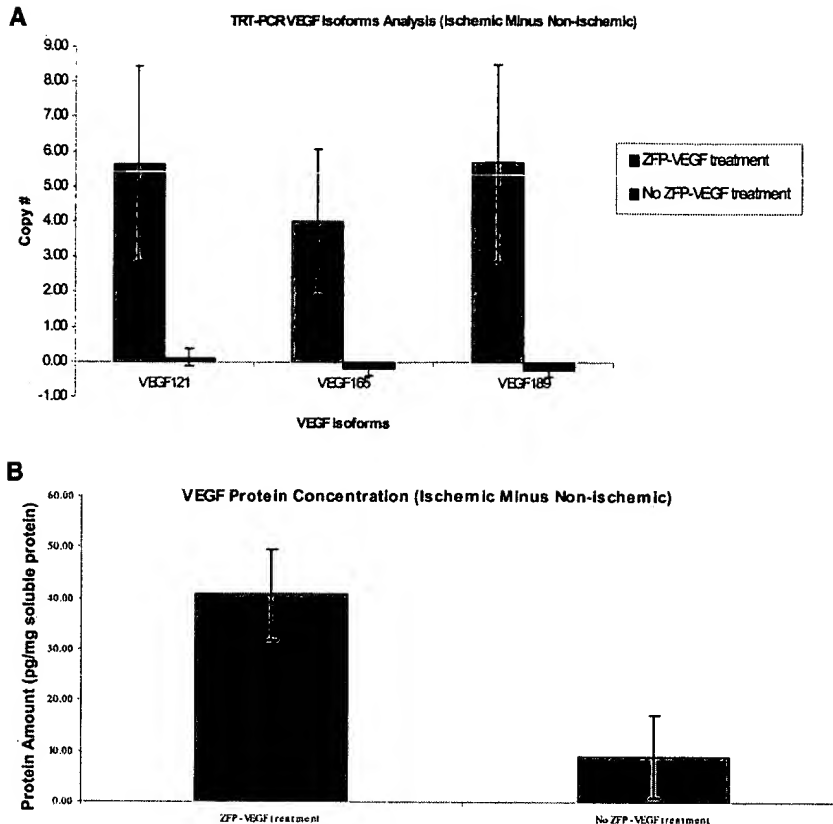
A

B

Figure 1. A, VEGF mRNA expression for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ by quantitative real-time RT-PCR in group 1 (day 3 after injection; n=6 in ZFP-VEGF–treated arm and n=7 in no-ZFP-VEGF–treatment arm). VEGF expression was measured as copy number of each isoform in ischemic leg minus that in nonischemic limb for each animal. Expression for all 3 mRNA isoforms was significantly higher ($P=0.05$, $P=0.05$, and $P=0.04$ for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, respectively) with ZFP-VEGF treatment compared with no ZFP-VEGF treatment. B, VEGF protein expression was measured by ELISA and was found to be significantly greater with ZFP-VEGF treatment compared with no ZFP-VEGF treatment ($P=0.02$). Abbreviations are as defined in text.

in the ZFP-VEGF-treatment arm (1.12 ± 0.42) compared with the no-ZFP-treatment arm (0.59 ± 0.39, $P<0.03$).

We then sought to determine whether changes in proliferation, cell death, or both contributed to the changes in vascular density in the ischemic muscle treated with the ZFP-VEGF plasmid. Representative samples of PCNA and TUNEL staining are shown in Figure 3. For quantitative assessment, the same 23 samples used in the vascular density studies were available. As shown in Figure 4A, the number of PCNA-positive cells in the ischemic TA muscle was significantly greater in the ZFP-VEGF-treatment arm ($3.11\pm1.10\%$) compared with the no-ZFP-treatment arm ($0.49\pm0.62\%$, $P<0.01$). Within the ZFP-VEGF treatment arm, the number of PCNA-positive cells in the ischemic limb was significantly greater than in the contralateral, nonischemic, noninjected TA muscle ($3.11\pm1.10\%$ versus $1.72\pm1.01\%$, $P<0.01$). As shown in Figure 4B, the number of TUNEL-positive nuclei in ischemic TA muscle was significantly lower in the ZFP-VEGF-treatment arm ($1.02\pm0.54\%$) compared with the no-ZFP-treatment arm ($1.90\pm0.68\%$, $P<0.01$). Within the ZFP-VEGF-treatment arm, the number of TUNEL-positive nuclei in the ischemic limb was similar to that in the contralateral, nonischemic, noninjected muscle.

Effects of ZFP-VEGF Plasmid at Later Times

Rabbits from group 3 (11 days after injection, 21 days of total ischemia), group 4 (21 days after injection, 32 days of total ischemia), and group 5 (21 days after injection, 32 days of total ischemia) underwent blood flow measurements before

injection and before sacrifice (Table 1). Perfusion in the ischemic limb was measured as a percentage of that in the nonischemic limb at both times. Immediately before injection, the mean perfusion ratio was $61.7\pm2.8\%$ for all rabbits, and there was no difference in these baseline values in either arm ($62.2\pm2.9\%$ for ZFP treatment versus $61.3\pm2.9\%$ for no ZFP treatment, $P=NS$). As shown in Figure 5A, the change in perfusion from 10 to 21 days was $6.57\pm1.52\%$ in the ZFP-treatment arm versus $3.38\pm0.87\%$ in the no-ZFP-treatment arm ($P<0.01$). As shown in Figure 5B, the change from 10 to 32 days was $13.15\pm1.77\%$ in the ZFP-treatment arm versus $6.13\pm1.55\%$ in the no-ZFP-treatment arm ($P<0.001$). As shown in Figure 5C, the change from 10 to 42 days was $20.16\pm2.84\%$ in the ZFP-VEGF-treatment arm versus $13.88\pm3.14\%$ in the no-ZFP-treatment arm ($P<0.01$). Qualitatively, similar results were obtained when changes in absolute perfusion values to the ischemic limb were used in place of the ratio. In groups 4 and 5, the number of α-smooth muscle actin–positive blood vessels per square millimeter was greater in the ZFP versus no-ZFP treatment arms (68.3 ± 37.0 versus 43.2 ± 21.1, $P=0.05$).

Discussion

Angiogenesis is a complex process whereby new blood vessels are formed from preexisting vascular structures.[6] Therapeutic angiogenesis seeks to exploit the phenomenon of angiogenesis to treat disorders of inadequate tissue perfusion, such as in patients with ischemic heart or PAOD.[7] VEGF has been and continues to be a leading candidate molecule for therapeutic angiogenesis. Although VEGF exists in multiple
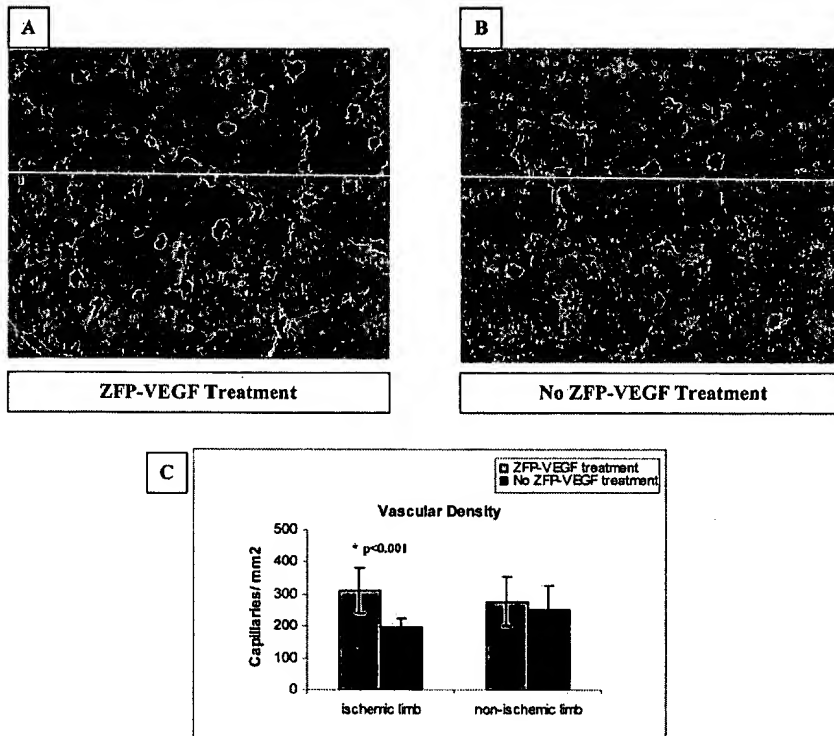
ZFP-VEGF Treatment

No ZFP-VEGF Treatment

Figure 2. Representative alkaline phosphatase stains show that capillary density in ischemic TA muscle with ZFP-VEGF treatment (A) was higher than with no ZFP-VEGF treatment (B) at 11 days after injection. Dark blue dots indicate capillaries (magnification ×200). C, Quantitative assessment of capillary density (capillaries/mm^2) in ischemic TA muscle (310 ± 71/mm^2) with ZFP-VEGF treatment was significantly higher than that of no ZFP-VEGF treatment (198 ± 25/mm^2). *$P<0.001$ vs ZFP-VEGF treatment. Abbreviations are as defined in text.

isoforms, approaches to achieve therapeutic angiogenesis in humans have exclusively used a single isoform of VEGF delivered as a protein, a plasmid-encoding cDNA, or an adenovirus.[12,14,22–24] The results from placebo-controlled trails with VEGF have been disappointing.[12,22] A method that would enhance the production of multiple VEGF isoforms has theoretical advantages over single-isoform therapy and thereby could overcome the limitations found in early human studies.

In this report, we sought to evaluate the effects of intramuscular injection of a plasmid DNA encoding a ZFP-VEGF–activating transcription factor in a preclinical model of PAOD. There were several notable findings. First, the ZFP-VEGF transcription factor led to upregulation of VEGF protein and 3 different VEGF isoforms in ischemic skeletal muscle. Second, treatment with the ZFP-VEGF plasmid led to an angiogenic response with an increase in capillary density, an increase in cellular proliferation, and a reduction in apoptosis in the ischemic TA muscle. Finally, there was evidence of a therapeutic angiogenic effect with an increase in perfusion to the ischemic limb in the ZFP-VEGF compared with the no-ZFP-VEGF treatment arms. These data demonstrate for the first time that modulation of endogenous VEGF gene expression and thereby, multiple VEGF isoforms can lead to therapeutic angiogenesis in a preclinical model of PAOD.

Zinc-finger transcription factors function by binding to specific DNA sequences in the regulatory region of a target gene. Rebar et al[16] found that the same ZFP-VEGF plasmid used in our current study was able to promote angiogenesis in a mouse ear. In ischemic muscle, endogenous transcription factors could have been present in the regulatory portion of the VEGF gene that contained the DNA sequences that

served as the binding site for the engineered transcription factor, and this would have resulted in the ZFP-VEGF not being able to increase VEGF mRNA or protein. This was not the case, and the ZFP-VEGF–activating transcription factor was able to increase VEGF gene expression in ischemic peripheral skeletal muscle.

Although the 3 VEGF isoforms examined in our report were upregulated at the mRNA level in ischemic muscle after ZFP-VEGF treatment, the increase in the VEGF$_{189}$ is particularly noteworthy. Differential splicing of the VEGF mRNA transcript results in isoforms that range in length from 121 to 206 amino acid residues.[8,9] The VEGF$_{121}$ isoform does not bind to the extracellular matrix and therefore, is freely soluble. VEGF$_{165}$ displays some heparin-binding properties and can also be detected in the circulation. VEGF$_{189}$ and VEGF$_{206}$ bind with high affinity to heparin sulfate proteoglycans in the extracellular matrix, which likely results in greater tissue retention of VEGF protein. It is interesting to speculate that an increase in expression of the higher-molecular-weight VEGF isoforms might yield better effects than either a single isoform or the lower-molecular-weight isoforms. This possibility is supported by recent work of Whitlock et al,[25] in which adenovirally mediated coexpression of 3 different VEGF splice variants (VEGF$_{121}$, VEGF$_{165}$, and VEGF$_{189}$) in 1 virus with a single insert encoding multiple isoforms or a mixture of 3 different viruses in a mouse model of hindlimb ischemia was superior in restoring blood than any one isoform alone. As previously noted, in a mouse ear model, Rebar et al[16] demonstrated that the same ZFP-VEGF–activating transcription factor used in our current report led to marked angiogenesis; however, unlike treatment with VEGF$_{164}$ (the murine variant) alone, the ZFP-VEGF plasmid–enhanced angiogenesis occurred without increasing vascular
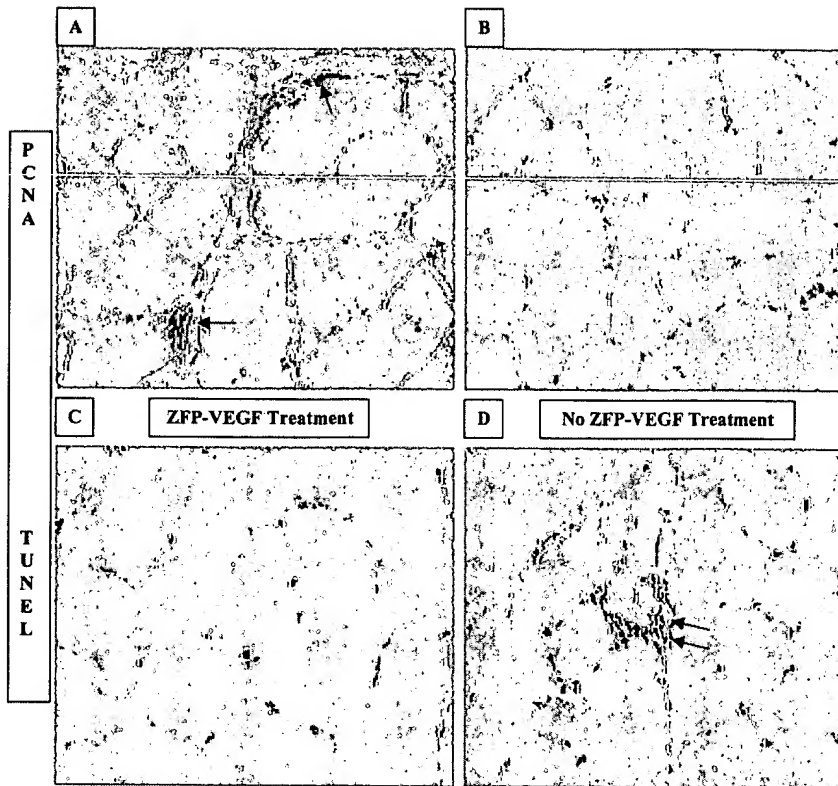
Figure 3. Representative examples of PCNA immunohistochemistry (A and B) and TUNEL staining (C and D) for labeling of proliferating and apoptotic nuclei from ischemic muscle from ZFP-VEGF-treatment (A and C) and no-ZFP-VEGF-treatment (B and D) arms at 11 days after injection. PCNA-positive cells (red, arrow) are visible in ischemic muscle with ZFP-VEGF treatment (A) but are rarely seen in ischemic muscle with no ZFP-VEGF treatment (B). TUNEL-positive nuclei (brown, arrow) are visible in ischemic muscle from both groups, but number of apoptotic cells in ZFP-VEGF-treated sample (C) was lower than that with no ZFP-VEGF treatment (D). Magnification, ×200 for all panels. Abbreviations are as defined in text.

leakiness. Vascular maturation is a complex process that involves the recruitment of nonendothelial support cells such as pericytes and vascular smooth muscle cells, both of which express smooth muscle actin.[26] In our report, treatment with the ZFP-VEGF-activating plasmid did result in an increase in the number of smooth muscle actin cells at 22 and 32 days after injection, although effects on vascular permeability after treatment of ischemic muscle were not examined.

We previously demonstrated that hindlimb ischemia results in a decrease in proliferation and an increase in apoptosis in ischemic muscle at 21 days after ligation, and the majority of apoptotic cells were endothelial.[10,19] In group 2 in our current study, we found that treatment of ischemic muscle with the ZFP-VEGF-activating transcription factor resulted in an increase in the fraction of PCNA-positive cells along with a decrease in the fraction of apoptotic cells. In the no-ZFP-VEGF-treatment arm in group 2, the majority of TUNEL-positive cells colocalized with endothelial cells (data not shown), and the fraction of apoptotic cells in the ZFP-VEGF-treatment arm was not different from that found in the nonischemic limb. This suggests an effect of VEGF on endothelial cells in ischemic skeletal muscle and confirms other reports wherein VEGF has been shown to act as a survival factor to protect endothelial cells from death.[27] Germani et al[28] showed that ex vivo treatment of myoblasts with a VEGF$_{165}$ adenovirus reduced apoptosis in vitro, and in a mouse hindlimb model, pretreatment with intramuscular adeno-VEGF$_{165}$ injection reduced apoptosis in muscle after ligation. The potential sequelae that could result from favorably modulating apoptosis in endothelial and/or other cells in ischemic peripheral skeletal muscle remain speculative.
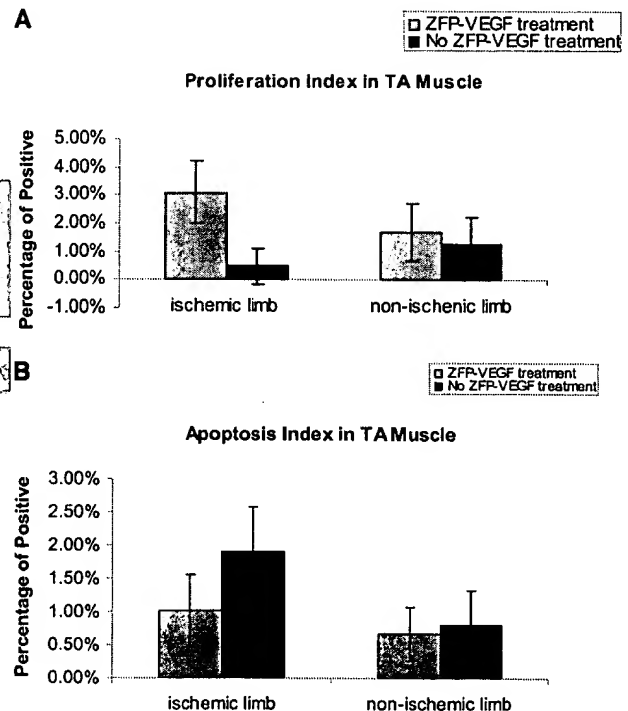

Figure 4. A and B, Results of quantitative assessments of PCNA and TUNEL staining, respectively. As shown in A, number of PCNA-positive cells in ischemic TA muscle was significantly greater in ZFP-VEGF-treatment arm (3.11±1.10%) than in no-ZFP-VEGF-treatment arm (0.49±0.62%, $P<0.01$ vs ZFP-VEGF treatment). As shown in B, number of TUNEL-positive nuclei in ischemic TA muscle with ZFP-VEGF treatment was significantly lower compared with that after no ZFP-VEGF treatment (1.02±0.54% vs 1.90±0.68%, $P<0.01$ vs ZFP-VEGF treatment arm). Abbreviations are as defined in text.

A

Change in Blood Flow Perfusion from Day 10 to 21

B

Change in Blood Flow Perfusion from Day 10 to 32

C

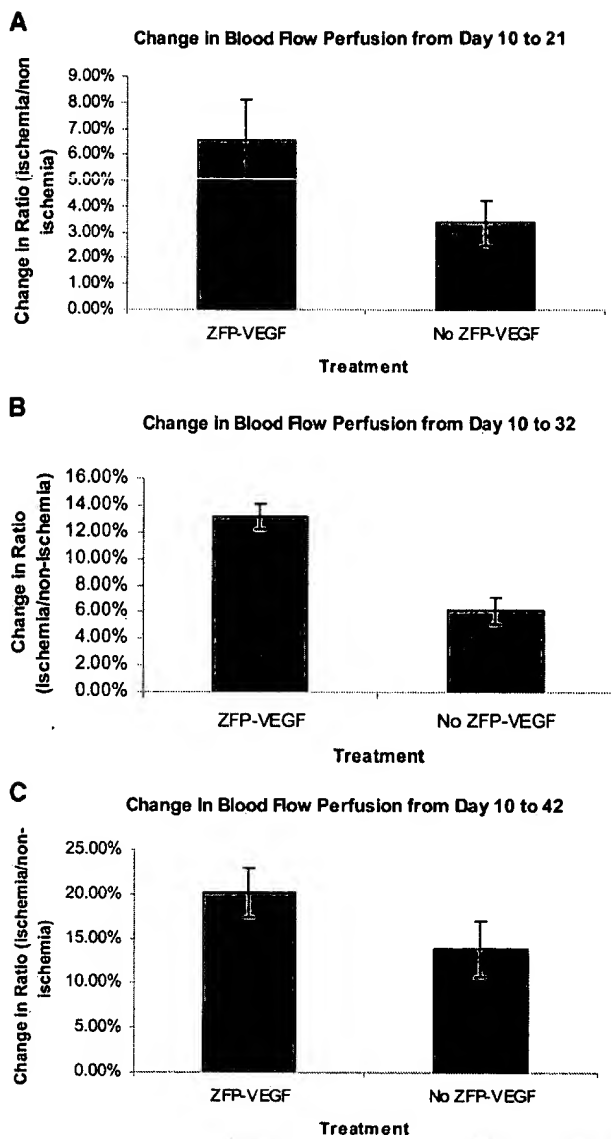Change in Blood Flow Perfusion from Day 10 to 42

Figure 5. Treatment of ischemic hindlimb muscle with ZFP-VEGF–activating transcription factor increased perfusion at multiple times. Animals underwent laser Doppler readings on day 10 (day of ZFP treatment or no ZFP treatment) and again at study termination after 21 (A), 32 (B), and 42 (C) days of ischemia. Change in blood flow to ischemic limb in ZFP-VEGF–treated arm was significantly greater than that in no-ZFP-VEGF treated arm at all times. Abbreviations are as defined in text.

Finally, we sought to determine whether ZFP-VEGF treatment would lead to therapeutic angiogenesis as measured by improvements in blood flow in the ischemic limb. By laser Doppler measurement, we found that ZFP-VEGF treatment resulted in a significantly greater increase in perfusion at all times measured (11, 22, and 32 days after injection) when compared with no ZFP-VEGF treatment. Although it is very difficult to compare studies in the same animal model from laboratory to laboratory, the results of our study certainly support the potential efficacy of the ZFP-VEGF–activating transcription factor. When given only 2 days after surgical ligation, Gowdak et al[29] showed that treatment with 1 mg $VEGF_{165}$ plasmid DNA formulated in liposomes led to

significant increases in blood flow in a rabbit hindlimb model by 11 days after treatment. When $VEGF_{165}$ was administered as an adenovirus 10 days after surgical ligation (the same time used in our report) and was compared with β-galactosidase, Vajanto et al[30] showed no significant increase in blood flow until the 30-day postinjection time in rabbits.

Our study suggests the potential of using ZFP-VEGF gene transfer to treat PAOD. Therapeutic modalities in humans must balance risks and benefits. ZFP transcription factors can be designed to target unique sites in the genome, and when studied in cell culture, they may regulate the intended target gene and no other.[31] The ZFP-VEGF used in our study targets a 9-bp sequence and is therefore predicted to have multiple potential binding sites. In cell culture, in addition to upregulation of the VEGF splice variants, other genes are upregulated at the mRNA level by microarray analysis, but VEGF is activated to the highest level.[16] The vector encoding the ZFP-VEGF–activating transcription factor could be modified with additional regulatory elements to permit changes in expression in response to intermittent stimuli (ie, hypoxia). The potential deleterious effects of prolonged VEGF expression in muscle must also be considered; however, the changes in VEGF expression from plasmid-based gene transfer are likely to be limited to period of only a few weeks.[32] likely to be limited to period of only a few weeks.[32]

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